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**TRANSGENIC PLANT PEPTIDE MEAT PROTECTION FROM
PATHOGEN AND SPOILAGE BACTERIA**

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Transgenic plant peptide meat protection from pathogen and spoilage bacteria.

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Abstract

The objective of this research project was to assess the potential of transgenic plant antimicrobial peptides as a novel method for inhibiting meat pathogen and spoilage bacteria. Our initial efforts included synthesizing the plant peptide genes from *Mirabilis jalapa* and *Allium cepa* known as *Mj-amp2* and *Ace-amp1*, respectively. Following some difficulty in the expression of functional peptides, we subsequently focused our efforts solely on the expression of the smaller antimicrobial peptide, Mj-AMP2. Following the identification of several key factors which hindered the expression of plant peptide genes in bacteria, we were able to successfully express the synthetic *Mj-amp2* gene in *Escherichia coli* and optimize inducer concentration and induction time required to produce significant amounts of biologically active proteins. With the ability to produce an active antimicrobial peptide in protein extracts isolated from the *E. coli* expression system we were able to determine whether the growth of specific foodborne pathogens could be inhibited by Mj-AMP2.

Executive summary

The objective of this project was to examine the potential of protecting meat spoilage and pathogen bacteria using plant antimicrobial peptides (AMPs). The initial phase of the project included a search of published plant peptide sequences with antimicrobial activity against foodborne pathogens and spoilage bacteria and synthesize genes that encode the peptides. It originally had been proposed that the antimicrobial activity of AMP genes would be tested by placing synthesized gene fragments coding for various AMPs into *Escherichia coli*, and later into GRAS status bacteria. It became clear that prior to transforming GRAS bacteria, the activity of the resulting peptides needed to be assayed using standard protocols and expression systems in *E. coli*. The peptides showing the highest antimicrobial activity against controls would then be used to assay against a panel of foodborne pathogens.

To date, antimicrobial peptides (AMPs) have been characterized from a number of different plant species, including the four o'clock plant, radish, onion and macadamia nuts (Woytowich and Khachatourians, 2001; Marcus et al., 1997; De Bolle et al., 1996; Cammue et al., 1992; Terras et al., 1992). The majority of these peptides belong to a group referred to as disulfide-linked peptides. They generally are approximately 50 amino acids in length and contain an even number of cysteine residues, which form disulfide bonds. It is the formation of these internal disulfide bridges that result in the necessary conformation of the molecules for biological activity. At the project's outset, we considered a number of different plant antimicrobial peptides for study, including Sn-1, Mj-AMP2, and Ace-AMP1. We subsequently narrowed our focus, and chose to synthesize and examine two antimicrobial peptides, Mj-AMP2 and Ace-AMP1, which are synthesized by the four o'clock and onion plants, respectively. These two plant peptides have been characterized and found to produce peptides of high antimicrobial activity against several plant pathogenic fungi and Gram positive bacteria (Cammue et al., 1992).

The *Mj-amp2* and *Ace-amp1* genes were designed and synthesized using different sets of overlapping oligonucleotides with appropriate flanking restriction enzyme cutting sites, and sequenced to ensure no mistakes had occurred during their synthesis. The AMP genes were cloned into expression vectors for controlled induction and expression of AMP peptides. At the point in the project where we failed to observe antimicrobial activity in assays conducted with protein extracts isolated from clones carrying the two different synthetic antimicrobial genes, it also became evident that almost no basic research on the synthesis of active peptides in prokaryotic expression systems was available. This in part reflects the complexities of expressing plant antimicrobial gene in bacteria. A review of the literature has revealed that to date there has only been one report of the successful *in vitro* expression and production of a plant antimicrobial peptide in a prokaryote expression system (Harrison et al., 1999). For this reason we felt it was necessary to determine the controlling factors which affect the expression of synthetic plant AMP genes in prokaryotes. We thus focused our efforts on *Mj-amp2* as it was the smaller and more efficacious of the two genes.

Our research on Mj-AMP2 focused on: 1) the presence of codon usages for certain amino acids that are found only rarely in bacteria, and 2) the formation of disulfide bridges necessary for peptide tertiary structure and hence activity. One problem with expressing *Mj-amp2* in *E. coli* is that eukaryotic genes, such as those of plants, contain codons for certain amino acids (i.e., arginine and isoleucine) that are produced in very low numbers in prokaryotes. Therefore, genes containing these codons are often produced in extremely low amounts or as truncated proteins that are non-functional. Examination of our gene sequence revealed the presence of two "rare" arginine codons. We therefore transformed our bacterial strains carrying the Mj-AMP2 expression vectors with a second vector that would code for the production of these rare amino acids. When a significant increase in antimicrobial activity was not observed, it became apparent that other factors were hindering peptide formation.

By employing a mutant strain of *E. coli* that enhanced the formation of disulfide bonds, we

were able to produce an AMP with increased antimicrobial activity against control organisms. Antimicrobial assays against the Gram positive bacteria, *Bacillus megaterium* ATCC 13632 and *Micrococcus luteus* ATCC 9342 which conventionally are used for bioassays of AMPs and have been performed under different conditions in order to determine optimal conditions for the expression of *Mj-amp2* in *E. coli*. Since *Mj-AMP2* is intracellularly synthesized, cells need to be treated so as to release the peptide, post-expression. Since most protocols use sonication as a cell lysis method, the effect that the time of sonication had on *AMP2* release was key to the successfully obtaining sufficient yield of transgenic plant AMP. Accordingly, when the *E. coli* cells were sonicated for 3 minutes, release of *AMP* and the percent inhibition of growth of *B. megaterium* and *M. luteus* was observed to be 46% and 73%, respectively.

Experiments were also undertaken to determine the concentration of the inducer IPTG required for optimal expression of the *Mj-AMP2* gene. The results from these assays indicated that as the concentration of IPTG was increased, a decrease in the *AMP2*-mediated inhibition occurred and that the highest percent inhibition of growth of *B. megaterium* and *M. luteus* occurred when 0.1 mM IPTG was used for induction. As well, the induction time required for *E. coli* AD494[pAE1] to produce concentrations of protein for maximum inhibition of control bacteria was also determined. We examined several time points, and determined maximum levels of *AMP2* production and hence inhibition of the growth of *B. megaterium* occurred between 7.5 and 15 minutes post induction, and this level of growth inhibition was sustained for up to 45 minutes after the addition of IPTG. However, one hour after induction, the percent inhibition of *B. megaterium* was observed to decrease. In the case of *M. luteus*, maximum levels of *AMP2* production, as measured by inhibition, were observed 15 minutes after induction and continued up to 45 minutes post induction.

Another factor examined in this study thought to affect the antimicrobial efficacy of *Mj-AMP2* was the type of media used in the assays. Cammue *et al.* (1992) previously reported that the activity of this peptide was inhibited by the presence of cations, thus we examined whether Luria Broth (LB), which contains 1% NaCl, negatively impacted the antimicrobial activity of *Mj-AMP2*. From assays carried out using different media, we were able to determine that the presence of 1% NaCl in the media had no effect on the growth inhibition of *M. luteus* or *B. megaterium*.

The work completed to date represents an essential first step for the expression of synthetic plant antimicrobial genes in a prokaryotic expression system. However, it is our opinion that this research has not yet fully explored the potential for plant *AMP* application as antimicrobial agents against food spoilage and pathogen bacteria, thus additional research remains to be conducted. Thus, while there is little doubt of the potential value of *AMPs* as antimicrobial agents, their value for application against food pathogens remains unclear. Now that some of the fundamental issues that limited the expression of plant *AMPs* in bacteria have been elucidated, it is anticipated that such tests may readily be performed using different *AMPs*, as originally intended. We will continue research in order to complete experiments that are in progress, as well as additional studies we feel are critical for publication of a manuscript.

Technical Report

Background

Defense-oriented proteins, referred to as pathogenesis-related proteins, play a direct role in controlling the growth of pathogens and are produced by mammals, insects, reptiles and plants. Since plants have been exposed to microbial pathogens over what might be termed "evolutionary time", they have evolved very effective mechanisms, such as antimicrobial peptides (AMPs), that restrict the growth and spread of microorganisms within their cells and tissues. Low molecular weight, cysteine-rich AMPs have been isolated and purified from several plant species such as *Amaranthus caudatus*, *Macadamia integrifolia*, *Raphanus sativus*, *Impatiens balsamina* and *Mirabilis jalapa* (Woytowich and Khachatourians, 2001). Many of these antimicrobial peptides have been analyzed by amino acid sequencing and their corresponding genes isolated and studied at the molecular level. AMPs are approximately 50 amino acids in length and contain an even number of cysteine residues (4, 6 or 8) that allow for the formation of disulfide bonds (Duvick et al., 1992). It is the formation of internal disulfide bridges that contribute to the high stability of AMPs while also dictating the final AMP conformation required for biological (antimicrobial) activity.

While these peptides appear to have evolved convergently (more than 150 different cationic peptide structures have been described) as evidenced by similar functional role and by a general high content of basic amino acids, the 3 dimensional structures of the active peptides are typically different (Hancock, 1997). This structural diversity includes β -sheets, α -helices, extended helices, and extended loops. In species such as insects (e.g., cecropin peptides) they represent an inducible system of defence against microbial pathogens, whereas in mammals (e.g., defensin peptides) they constitute the predominant protein within neutrophils (Evans and Harmon, 1995).

Bacterial Outer Membranes and Antimicrobial Peptides

The Gram negative bacteria constitute the largest problem to the food industry in terms of potential pathogens, as well as predominant spoilage bacteria. The outer membrane of the gram negative bacteria is only selectively permeable to antimicrobials, a consequence of the limiting size of porin channels. Hancock (1997) summarized these effects by indicating that porin channels represent <5% of the surface area of bacteria such as *E. coli*, and since antibiotics such as the β -lactams approach the size exclusion range of porin channels, they are hindered as a consequence of intermolecular interactions with the amino acids lining the channel. This effect alone may reduce the potential uptake of β -lactams by *E. coli* by a factor of 1000. Attached organisms, or other organisms with high intrinsic resistances to antimicrobial treatment commonly reduce permeation rates another 100 fold. Other mechanisms of uptake, including self-promotion, are active for certain antimicrobial agents. However, it is apparent from an abundance of research that microorganisms initially resist antimicrobial compounds primarily by the selective nature of their cell membrane. It also follows that irreversible damage to this membrane can render a potential pathogen incapable of regulating its internal ion concentration, and hence the dissipation of trans-membrane ion gradients necessary for ATP synthesis. Furthermore, increased permeabilization of the cell membrane enhances the potential for combinatorial use of antimicrobial compounds of interest to the food industry along with membrane permeabilizing peptide agents.

A rather complete picture of how cationic peptides interact with cell membranes now exists, having been described for neutrophil-derived proteins (indolicidins) (Lehrer et al., 1990). As occurs for many polycations, indolicidins cross the cell membrane as a consequence of self-promotion, a

process involving the displacement of Ca^{2+} - or Mg^{2+} -divalent ions from their binding sites on the surface lipopolysaccharides (LPS). Being larger than the divalent cations, the peptides distort the outer membrane structure once inserted into the membrane. A transmembrane electrochemical potential of ~ 80 mV is required to facilitate the passage of indolicidins through the periplasm into the cytoplasmic membrane. Once inserted, a channel is formed through which cytoplasmic constituents may leak. The size of channels formed by various peptides is variable. The binding of the peptides to LPS explains, in part, the tendency for some peptides to exhibit anti-endotoxin activity. Permeabilization of the outer membrane is also consistent with the observation that peptides tend to increase penetration of probe tracers into the cell cytoplasm, a feature which also results in synergistic interactions with other known antibiotics (Vaara and Porro, 1996). Lastly, the observation that development of resistant mutants occurs infrequently reflects the unlikely chance that a mutation will lead to an altered cell membrane, a mutation far more complex than that of an altered enzyme.

Plant Antimicrobial Peptides

As with animals, plants have evolved highly effective mechanisms to protect themselves from invasion and degradation by pathogenic bacteria. Upon recognition of certain microbial signals, plants produce various antimicrobial substances, including peptides. Alternatively, these antimicrobial substances are produced constitutively in specialized cells. The following families of plant antimicrobial peptides are summarized in the Table 1.

Table 1: Families of plant antimicrobial peptides found in plants and their general characteristics.

Peptide	Size (# aa-s)	Target Microbes*	Adverse effects on plant or animals
Thionins	45 - 47	Fungi G+ bacteria G- bacteria	Animals Insects Plants
Plant Defensins	45 - 54	Fungi	Animals (inhibit α -amylases)
Lipid Transfer Proteins	90 - 93	Fungi G+ bacteria G- bacteria	Animals (inhibit α -amylases)
Hevein- and Knottin-Type Peptides	Variable most ~ 40	Fungi G+ bacteria	Animals (inhibit α -amylases)
Four Cysteine-Type Peptides	20 - 33	Fungi G+ bacteria Some G- bacteria	

*Demonstrated antimicrobial activity. Although the exact action of the different plant peptides is not well characterized, all antimicrobial peptides studied so far appear to damage the plasma membrane of the target microorganisms by permeabilization of cytoplasmic membrane, such as through interactions with membrane phospholipids.

Presently, those plant peptides with potential value as antimicrobial agents are characterized as being short (<60 amino acids in length), have an even number of cysteine residues (4, 6, or 8) which pair via disulfide bridges, and apparently exert their antimicrobial effects at the level of the microbial plasma membrane. All have a net positive charge of at least +2 (most 4, 5, and 6) as a consequence of the occurrence of lysine, arginine, or modified amino acids. At neutral pH, these compounds have a positive charge and an amphipathic 3-D structure; a consequence of having a hydrophobic region

comprised of non-polar amino acid side chains and a hydrophilic region where polar and positively-charged amino acids are positioned. As stated earlier, these peptides vary in length, sequence, and secondary structure. The >150 currently-known natural cationic peptides which meet the above criteria may be positioned in the four general peptide classes described above (Table 1).

The best characterized peptides are those for which the genes and regulatory elements coding for the peptides are known. While the state of knowledge in this research area is undergoing continual growth, peptides from the *Mirabilis* and *Macadamia* genera represent some of the best characterized systems.

A number of other peptides have been purified and characterized and while the putative amino acid sequences have been determined, the genetic sequences have not yet been elucidated. For example, peptides of the thionin class (cysteine- and lysine-rich polypeptides) have been isolated from monocot seeds, including wheat, rye, barley, sorghum and oats; some dicots (i.e., mistletoe and Abyssinian cabbage) also produce thionin-like peptides. Novel antimicrobial peptides (33 residues) which bear no homology to the thionins have also been isolated from corn kernels (Duvick et al., 1992). There has also been developments in the area of the design and synthesis of synthetic antimicrobial peptides with, for example, increased amphipathicity (Dykes et al., 1998; Powell et al., 1995).

Production of antimicrobial peptides for industry

At present, naturally produced peptides have not proven economically viable, with the rather significant exception of nisin which is produced in large quantities by *Lactococcus lactis* via industrial fermentation. Alternate strategies for obtaining peptide compounds include protein chemical synthesis, and recombinant DNA biotechnology. Chemical synthesis of these relatively short peptides is relatively straight-forward, involving the linkage of individual amino acids from the N- to the C-terminus using solid-phase automated peptide-synthesis equipment. While this approach has certain advantages, including the fact that non-natural stereo isomers (D-isomers) or modified amino acids can be added to the formulation to enhance efficacy and minimize resistant strains, it is expensive and isn't suitable for producing large quantities of peptide or scale-up (Marcus et al., 1997), and problems exist when synthesizing peptides with high numbers of arginine or tryptophan residues. In contrast, recombinant molecular biology methods for obtaining peptides are much more viable, less expensive, and involve a final fermentation step as in traditional antibiotic production. However, for this to be a viable strategy, those factors that govern the expression of the properly folded plant protein in bacterial systems must be delineated. For these reasons, a significant portion of this study was invested to determine the necessary conditions for the expression of *Mj-amp2* in *E.coli*. Once established, antimicrobial assays using the plant AMP, *Mj-AMP2*, against several foodborne pathogens were performed.

Mj-AMP2, an antimicrobial peptide isolated and purified from the seeds of *M. jalapa*, is highly basic and consists of 36 amino acids. It contains six cysteine residues that are involved in disulfide bridge formation and *in vitro* assays have demonstrated that it is a potent inhibitor of several plant pathogenic fungi and some Gram positive bacteria (Cammue et al., 1992; Broekaert et al., 1997).

Our original study goal was to examine the potential of plant AMPs for protecting meat and its products from bacterial contamination. As a logical first step towards this objective, a thorough search the literature on isolation and purification plant peptides and their corresponding genes was conducted to identify those AMPs which had potential for antimicrobial activity against foodborne pathogens. These AMP sequences would then be synthesized and cloned into a prokaryotic expression system for subsequent testing against a number of foodborne pathogenic microorganisms. We are in the final stages of meeting our original objective, but a series of technical problems encountered in expressing a functional plant peptide from our synthetic AMP genes in *E. coli* necessitated expanding our goals to

include defining the ideal conditions required for the expression of the *M. jalapa* *Mj-amp2* gene in a prokaryotic expression system.

Results and Discussion

Synthesis of antimicrobial genes

From the analysis of the published reports it was concluded that three peptides, produced by *M. jalapa*, *Allium cepa* and *Solanum tuberosum*, would meet our requirements of: 1) antimicrobial activity against bacteria, 2) small size and 3) available and published gene or amino acid sequence. As illustrated in Figures 1 A-C and 2, the antimicrobial peptide genes were synthesized using a series of oligonucleotides with complimentary overlapping regions that were annealed to each other and then amplified via the polymerase chain reaction (PCR). Restriction enzymes sites, which were required for subsequent cloning of the synthetic genes into the expression vectors, were also introduced by PCR. Initial studies with *Mj-AMP2* included both the peptide with signal sequence, as well as the mature peptide alone. The synthesized genes for *Ace-AMP* and *Sn-AMP* were designed so as to only express the mature peptide. The PCR-amplified genes were then digested with the specific restriction enzymes and cloned into either pSE380 (Figures 3 and 4) or pGEX-5x-3. The resulting clones were then screened for the presence of the synthesized gene, and sequenced to ensure that they contained the complete gene sequence and no mutations had occurred during the PCR amplification (Figures 5 and 6). Sequencing performed on a number of clones revealed that *Mj-amp2* and *Ace-amp1* were successfully synthesized, but that all the *Sn-amp* gene clones had either missing or additional bases. It was decided to restrict our activities to the synthesis and subsequent assay of *Mj-AMP2* and *Ace-AMP1* peptides for antimicrobial activity.

Assaying *Mj-AMP2* and *Ace-AMP1* for antimicrobial activity

Following oligonucleotide synthesis, synthetic *Mj-amp2* and *Ace-AMP* genes were cloned into an expression vector. *Mj-amp2* was cloned into both pGEX-5x-3 (a Glutathione S-Transferase [GST] based protein fusion vector under IPTG inducible control) and pSE380 (also IPTG inducible). *Ace-AMP* was cloned into pSE380 only (Figures 1B and 4). A number of studies were performed with both the leader sequence and peptide coding regions included in pGEX-5x-3 (Figures 1A, 5 and 6), however we further restricted our research to the mature peptide sequence using pSE380 as an expression system. The reasons for this were mainly based on the lack of antimicrobial activity found following cleavage of the gene product from the GST-fusion protein complex, as well as the expense of the pGEX-5x-3 kit reagents. Plate assays undertaken using crude *Mj-AMP2* protein extract isolated from *E. coli* strains DH5a and BL21 transformed with *Mj-amp2* inserted in pSE380 did not display any antimicrobial activity against the control bacteria *Bacillus megaterium* and *Micrococcus luteus*. Since previous studies by Cammue *et al.* (1992) indicated that *Mj-amp2* antimicrobial activity was adversely affected by the presence of divalent cations, desalting columns were used to treat the cellular extracts prior to analysis of antimicrobial activity. Unfortunately, assays performed using desalted protein extracts, different media, and varying concentrations of the inducer IPTG did not lead to significant antimicrobial activity against *B. megaterium*. It was somewhat surprising that similar experiments performed using desalted crude extracts from *Ace-amp1* clone did not display any antimicrobial activity, as previous studies on the purified peptide indicated that it was only weakly affected by physiological concentrations of inorganic cations. The negative results we had been obtaining up until this point clearly indicated that something, other than the presence of cations, was adversely affecting the activity of these peptides *in vitro*.

Mj-AMP2 peptide gene sequence

```

5' ATG GCT AAG GTT CCA ATT GCC TTT CTC AAA TTC GTC ATC
3' TAC CGA TTC CAA GGT TAA CGG AAA GAG TTT AAG CAG TAG

GTG TTG ATT CTC TTC ATT GCC ATG TCA GGC ATG ATA GAA
CAC AAC TAA GAG AAG TAA CGG TAC AGT CCG TAC TAT CTT
      mature peptide starts here
GCA TGC ATA GGA [REDACTED] AAC GAG AAC GTG
CGT ACG TAT CCT [REDACTED] TTG CTC TTG CAC

GGC CCA CCA TAC TGC TGT TCG GGT TTC TGC CTC CGT CAA
CCG GGT GGT ATG ACG ACA AGC CCA AAG ACG GAG GCA GTT

CCT AAC CAA GGT TAC GGT GTT TGC AGG AAC CGC TAA 3'
GGA TTG GTT CCA ATG CCA CAA ACG TCC TTG GCG ATT 5'
  
```

Amino acid sequence: MAKVPIAFLKFVIVLILFIAMSGMIEACIGNGGRCNENVGPPY
CCSGECLRQPNQGYGVCRNB

Oligo #1

Oligo #2

[REDACTED] Overhang for annealing

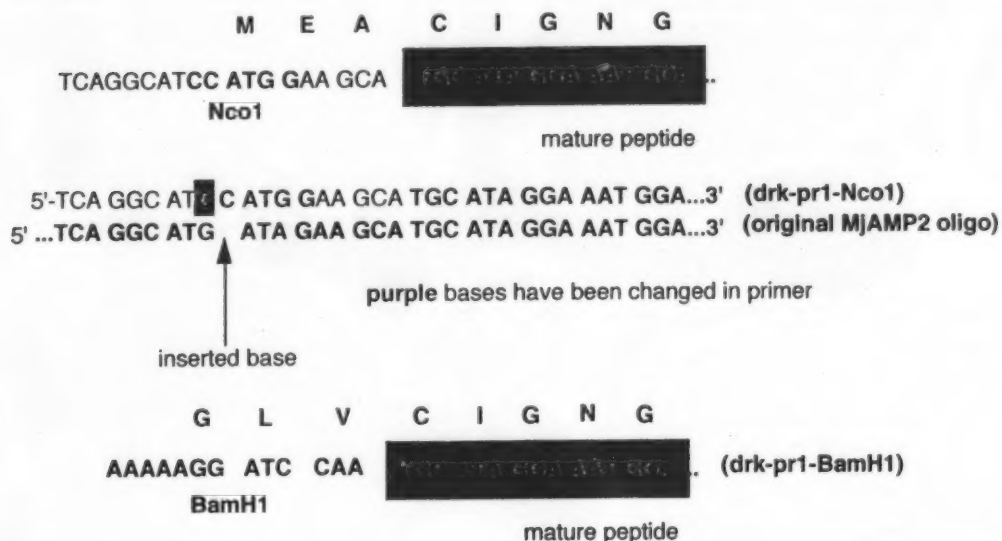


Figure 1A. Sequence of the *Mj-amp2* gene, including signal sequence and mature peptide, illustrating how two oligonucleotides were synthesized with overlapping complimentary regions (see yellow and pink regions). The 15 base overhang region is highlighted in purple for identification. The DNA coding region of the mature peptide is indicated by the black arrow; the corresponding amino acid sequence (see appendix A for amino acid key) is included and is underlined. To facilitate cloning of only the mature Mj-AMP2 gene into either pGEX-5x-3 or pSE380, PCR reactions were conducted using either primers that would introduce either a *Bam*H I site or a *Nco* I site (primer sequences shown at bottom).

BamHI NcoI

5' AAAAAAGGATCCCC ATG GGA 20
 3' Met Gly

5' CAG AAC ATA TGC CCA AGG GTT AAT CGA ATT GTG ACA CCC TGT GTG 65
 3' GTC TTG TAT ACG GGT TCC CAA TTA GCT TAA CAC TGT GGG ACA CAC

GCC TAC GGA CTC GGA AGG GCA CCA ATC GCC CCA TGC TGC AGA GCC 110
 CGG ATG CCT GAG CCT TCC CGT GGT TAG CGG GGT ACG ACG TCT CGG

CTG AAC GAT CTA CGG TTT GTG AAT ACT AGA AAC CTA CGA CGT GCT 155
 GAC TTG CTA GAT GCC AAA CAC TTA TGA TCT TTG GAT GCT GCA CGA

GCA TGC CGC TGC CTC GTA GGG GTA GTG AAC CGG AAC CCC GGT CTG 200
 CGT ACG GCG ACG GAG CAT CCC CAT CAC TTG GCC TTG GGG CCA GAC

AGA CGA AAC CCT AGA TTT CAG AAC ATT CCT CGT GAT TGT CGC AAC 245
 TCT GCT TTG GGA TCT AAA GTC TTG TAA GGA GCA CTA ACA GCG TTG

ACC TTT GTT CGT CCC TTC TGG TGG CGT CCA AGA ATT CAA TGC GGC 290
 TGG AAA CAA GCA GGG AAG ACC ACC GCA GGT TCT TAA GTT ACG CCG

AGG ATT AAC 3' 299
 TCC TAA TTG 5'

Ace-AMP1 mature peptide - gene sequence

Amino acid sequence:

MGQNICPRVNRIVTPCVAYGLGRAPIAPCCRALNDLRFVNTNRNLRRACRCLVGVVNRNPGLRRNPRF
 QNIPRDTFVRPFWWRPRIQCGRIN

Ace-dk1

Ace-dk3

Ace-dk2

Ace-dk4

total PCR product 299 + 18 = 317

5'- TAT TTA GTC GAC CCC GGG GTT AAT CCT GCC -3'

Drk pr13 reverse primer with Sma1/Sal1 sites. anneals to the sense strand (see underlined codons)

Figure 1B. Sequence of the *Ace-amp1* gene (mature peptide-coding region only) illustrating the 4 overlapping oligonucleotides that were synthesized (see Ace-dk1 - Ace-dk4). The amino acid sequence is also included (see appendix A for amino acid key).

```

          BamHI      NcoI
5' AAAAAAGGATCCCC ATG GGA 20
3' TTTTTT CCTAGGGG TAC CCT

GGT TCA AAT TTT TGT GAT TCA AAG TGC AAG CTG AGA TGT TCA AAG 65
CCA AGT TTA AAA ACA CTA AGT TTC ACG TTC GAC TCT ACA AGT TTC

GCA GGA CTT GCA GAC AGA TGC86 TTA AAG TAC TGT GGA GTT TGT TGT 110
CGT CCT GAA CGT CTG TCT ACG AAT TTC ATG ACA CCT CAA ACA ACA

GAA GAA TGC AAA TGT GTG CCT TCT GGA ACT TAT [REDACTED] 155
CTT CTT ACG TTT ACA CAC GGA AGA CCT TGA ATA CCA TTG TTT GTA

[REDACTED] 200
CTT87 ACA GGA ACA ATA TCC CTG TTC TTC TTG AGA TTC CCG TTC AGA

[REDACTED]69 3' 212
TTT ACG GGA ACT 5'

```

SN1 snakin-1 peptide - gene sequence

Sn-dk1

Sn-dk2

Sn-dk3

TAT TTA GTC GAC CCC GGG TCA AGG GCA TTT AGA
Sma 1

SN-dkpr2
antisense primer (binds sense strand)

Figure 1C. Sequence of the *SN-1* gene (mature peptide-coding region only) showing the 3 oligonucleotides with complementary overlap regions (see Sn-dk1-Sn-dk3). The first 20 bases code for the restriction sites *Bam*HI and *Nco*I, allowing insertion of this construct into either pGEX-5x-3 or pSE380. Note that the reverse primer codes for the restriction enzyme *Sma*I. The *Sma*I site was used for all the AMP genes in this project.

Polyacrylamide gel showing crude preparation of oligonucleotides for peptides Mj-AMP2 and Ace-AMP1.

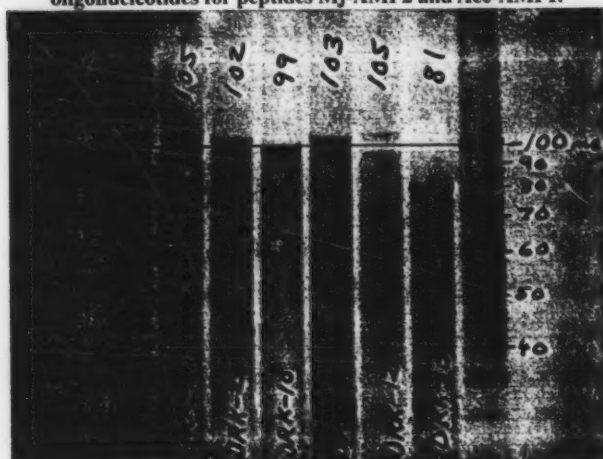


Figure 2. Polyacrylamide gel showing synthesized oligonucleotides corresponding to overlapping fragments of the *Mj-amp2* and *Ace-amp1* genes, as represented schematically in Figures 1A and 1B.

Figure 4. Diagram of the pSE380 plasmid with details of restriction sites and resistance gene markers. The plasmid permits IPTG-inducible expression from the *trp-lac* promoter. The plasmid was cut the *Nco* I and *Sma* I restriction enzymes and ligated with the *Mj-amp2* gene.

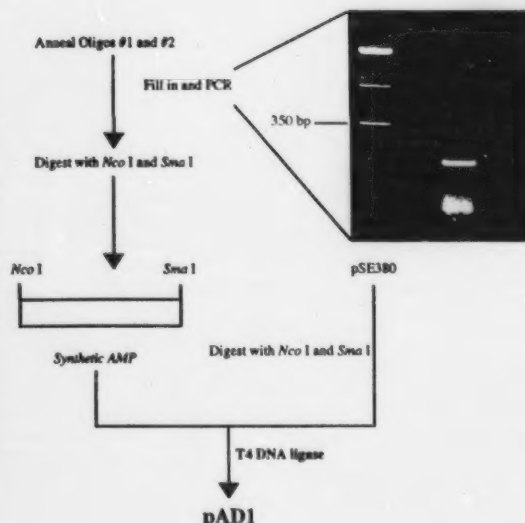
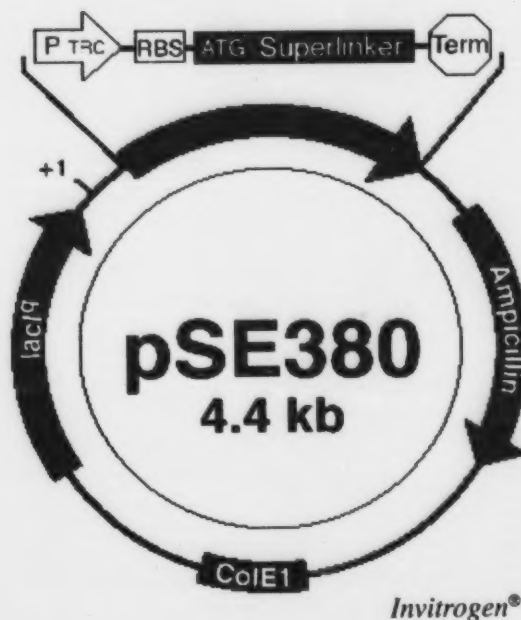
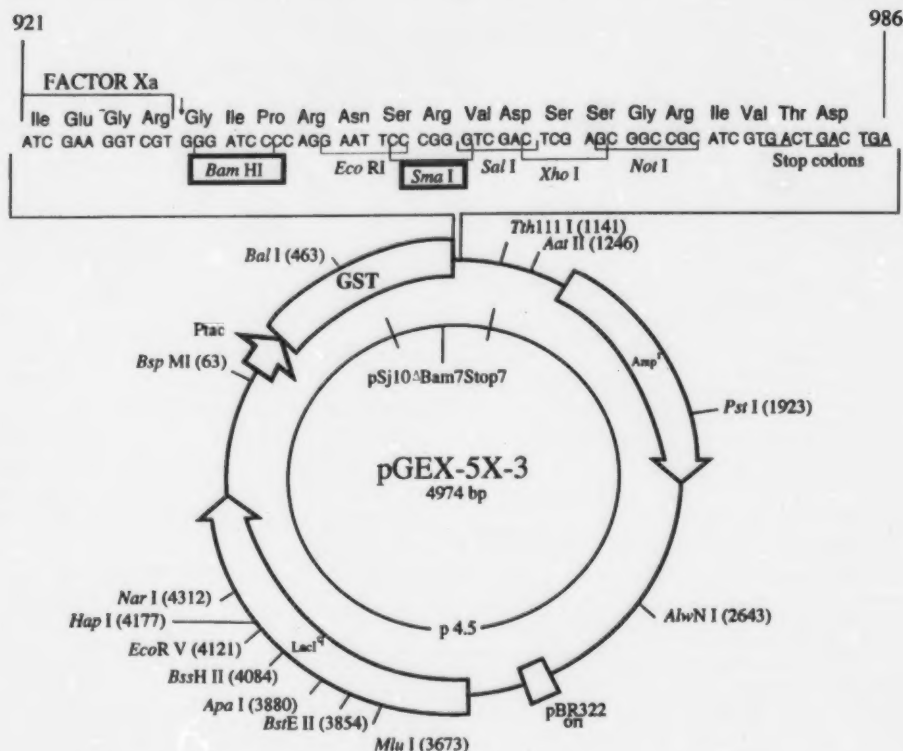


Figure 3. Construction of the *Mj-amp2* gene and subsequent cloning into the expression vector pSE380. Inset shows PCR amplification of the ~150 bp fragment (single intense band), as well as the 50 bp molecular weight ladder (left lane).

Superlinker

Enz	Position
Nco I	275
Dra III	294
Bsm I	304
BstB I	306
EcoR I	308
BspM I	326
Sal I	332
Aat II	333
Xma I	353
Sma I	355
BamH I	389
Bgl II	396
Eco47 III	420
Nhe I	438
Xba I	450
Kpn I	460
Nae I	478
SnaB I	484
Stu I	507
Avr II	508
Eag I	524
Not I	524
Pst I	535
Sac II	569
Xho I	584
Sac I	585
Spe I	594
Hind III	622



GST - glutathione S-transferase

Figure 5. Diagram of the pGEX-5x-3 plasmid with detailed map of restriction sites and resistance genes. As highlighted in color, the *Mj-amp2* gene was inserted between *BamH* I and *Sma* I restriction sites.

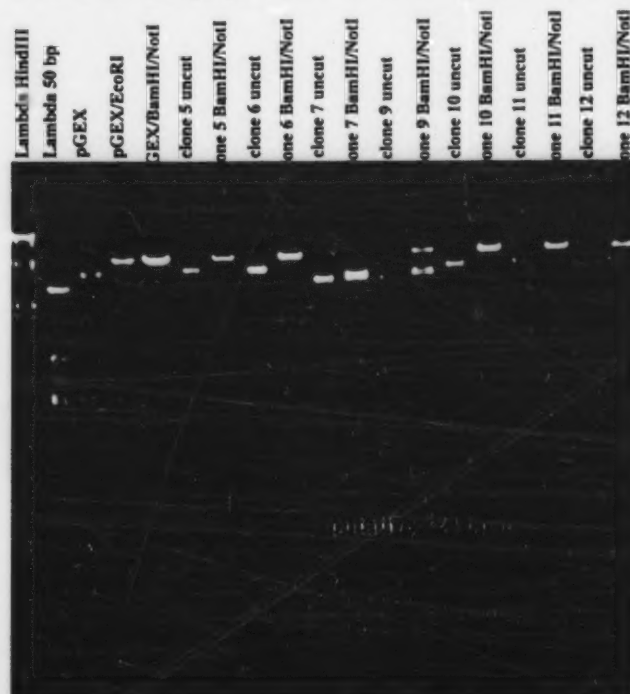


Figure 6. Agarose gel used to screen a number of *E. coli* BL21 clones transformed with the pGEX-5x-3 plasmid containing the synthetic *Mj-amp2* gene (coding for the mature peptide, as well as the signal sequence). If the *Mj-amp2* gene had successfully been inserted, then digestion with *BamH* I and *Not* I restriction enzymes would have resulted in the release a small fragment (224 bp) of DNA. Using this procedure, clones 5, 9, 11 and 12 were chosen for sequencing to confirm the presence and sequence of the *Mj-amp* gene.

A literature search on the *in vitro* expression of plant antimicrobial peptides revealed that there have only been two reports of the successful expression and production of plant antimicrobial peptides. The first one, which involved a radish defensin (Vilas Alves et al., 1994), was produced in yeast which is a eukaryotic system. The second one, involving a gene isolated from *Macadamia integrifolia*, was expressed using an *E. coli* expression system in conjunction with the plasmid pSB161 (Harrison et al., 1999). Previous studies have shown that the expression of eukaryotic genes in prokaryotic expression systems can be impeded by differences in codon usage, especially arginine codons where two of six variations in the triplet are very poorly represented in *E. coli* tRNAs (Spanjaard et al., 1990). The plasmid pSB161 compensates for these rare tRNAs as it contains the *argU* gene. When we examined the coding sequences of our AMP genes, it was found that these rare codons do exist. We therefore needed to eliminate this as a factor that might affect the production of functional AMPs in our *E. coli* strains, since either a low amount of the peptide being produced, or the production of truncated versions of the peptides would both hinder determining peptide antimicrobial activity. In order to eliminate altered codon usage as a problem, we obtained a series of rare *argU* encoding vectors (pSBETa, b and c) from Dr. Steinbiss (Max Planck-Inst. Fuer Zuechtungsforschung Abt. Genetische Grundlagen der Pflanzenzuechtung, Germany) and co-transformed Mj-amp2[pSE380] with these vectors.

Plate assays using extracts from strains containing Mj-amp2 and the *argU* gene vectors were subsequently performed against the control microorganisms, *M. luteus* and *B. megaterium*. Although no inhibition against *B. megaterium* was observed, a partial 8 mm zone of influence was seen when crude Mj-AMP2 was assayed against *M. luteus* (data not shown). If the presence of rare arginine codons were the only factor responsible for the lack antimicrobial activity, then control plate assays should have shown a clear zone of inhibition due to the fact that Mj-amp2 only contains two rare arginine codons. We therefore concluded that while the lack of antimicrobial activity may partially have been due to the lack of rare arginine codons in *E. coli*, a problem with the peptide assuming the proper conformation, or folding pattern, was also involved (due to the presence of cysteine-cysteine disulfide bonds).

One significant factors complicating the production of active plant antimicrobial peptides in prokaryotes lies in the fact that all plant AMPs characterized to date contain multiple disulfide bridges that must undergo proper folding to be active. Moreover, *E. coli* cytoplasmic proteins generally do not contain structural disulfide bonds, therefore Mj-amp2 was probably not forming the specific structure required for antimicrobial activity. Early studies by Derman and Beckwith (1995) demonstrated that *E. coli* alkaline phosphatase localized in the cytoplasm slowly acquired enzymatic activity if cell growth was suspended over a period of six hours. From their findings, it was found that the mechanism responsible for reducing cytoplasmic proteins was no longer functioning in cells that had ceased to grow. It was proposed and demonstrated that in order for disulfide bonds to form in the cytoplasm *E. coli*, the gene for the enzyme thioreductase, *trxB*, must be inactivated. When antibacterial assays were performed using proteins extracted from *E. coli* DH5 α [Mj-amp2], we observed similar findings. Protein extracts from cells that were still growing didn't inhibit the growth of *B. megaterium* or *M. luteus*. However, when the cells were washed with buffer and incubated on ice for five hours prior to protein extraction, approximately 10% growth inhibition against *B. megaterium* and *M. luteus* was observed with titer plate assays (data not shown).

From experiments undertaken, it appeared that one reason for the lack of antimicrobial activity from extracts isolated from *E. coli* DH5 α [Mj-amp2] was that disulfide bond formation was not occurring *in vivo*. To confirm this, we transferred the Mj-amp2 construct, known as pAE1, to the *E. coli* strain AD494. *E. coli* AD494 contains a thioredoxin reductase (*trxB*) mutation which allows the formation of disulfide bonds within the cytoplasm of the cell. When microtiter plate assays using *B. megaterium* and *M. luteus* as controls were undertaken using crude protein extracts from *E. coli* AD494[pAE1], a

significant increase in percent inhibition was observed. Since a number of factors have previously been shown to impact the expression of efficacious synthetic antimicrobial peptides, a more fundamental knowledge of the controlling factors for efficacious peptide expression was now necessary. This step was not performed previously as the effects are typically quantified in terms of altered antimicrobial activity, and earlier results failed to yield AMPs with significant activity. Thus, a series of experiments that involve assaying Mj-AMP2 against the positive control microorganisms, *B. megaterium* and *M. luteus*, under different conditions were undertaken to determine the ideal parameters are for maximum expression.

The effect sonication on the inhibition of *B. megaterium* and *M. luteus*

One of the first parameters examined was the effect of sonication time on the inhibition of the test microorganisms. It was determined that when sonication times of 0.5 and 1 minute were applied, no difference in percent inhibition was observed (Figure 7). However, when the *E. coli* cells were sonicated for 3 minutes, it was found that the percent inhibition of growth of *B. megaterium* and *M. luteus* increased to 46% and 73%, respectively. Examination of the absorbencies of the test strains after 17 hours of incubation with the crude protein extracts revealed that the samples containing extracts from cells that had been sonicated for 3 minutes had OD₆₀₀ readings two times lower for *B. megaterium*, and four times lower for *M. luteus*, than the controls. These results clearly indicated that sonication times of less than three minutes failed to release sufficient antimicrobial peptide for significant growth inhibition of the control strains. Therefore, subsequent assays done on foodborne pathogens must use protein extracts that were sonicated long enough for the peptides to be released into the extraction buffer.

Determination of optimal induction time and concentration for expression of Mj-AMP2

Since the *Mj-amp2* gene was cloned into a vector under inducible control, we conducted a series of experiments to determine the optimal induction time for *E. coli* AD494[pAE1] in order to produce maximum levels of antimicrobial activity. The first time point studied was 1.5 minutes since it takes approximately this long for one round of translation to occur. To ensure that translation was halted at the desired time points to be studied the antibiotic chloramphenicol, which inhibits protein

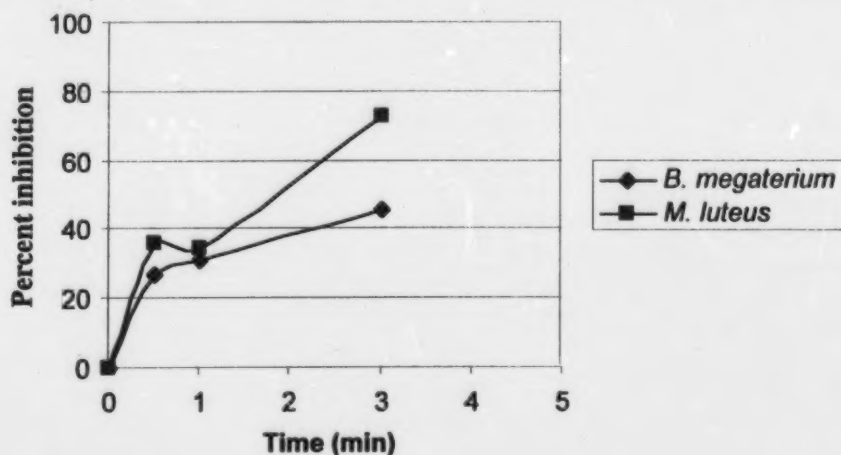


Figure 7. The effect of sonication time on the percent inhibition of control bacterial strains. *E. coli* strain AD494[pAE1] cells were sonicated for different lengths of time, after which a 200 ml aliquot of crude Mj-AMP2 protein extract isolated from each preparation was used to challenge either *B. megaterium* or *M. luteus*. *E. coli* cells were induced for one hour with 0.2 mM IPTG.

synthesis, was added to the 100-ml cultures to a final concentration of 16 mg/ml. To ensure that any inhibition of growth of the test microorganisms observed was not due to the presence of residual chloramphenicol the AMP-producing *E. coli* cells were centrifuged and washed twice. Our results indicated that not enough protein was produced after 1.5 minutes of induction to inhibit the growth of *B. megaterium* or *M. luteus*, as illustrated in Figure 8. Maximum levels of inhibition of the growth of *B. megaterium* were reached, however, between 7.5 and 15 minutes post induction and this level of growth inhibition was observed up to 45 minutes after the addition of IPTG. However, one hour after induction the percent inhibition of *B. megaterium* decreased to approximately 28% from a maximum of 57% reached at 15 minutes. In the case of *M. luteus* it was also found that 1.5 minutes of induction was insufficient for growth inhibition. Although 27% inhibition was observed after 7.5 minutes, it was not until 15 minutes after induction that maximum levels of inhibition were observed. These high levels of growth inhibition were observed up to 45 minutes post induction. When an induction time of 90 minutes was tested, similar results were observed with *M. luteus* and *B. megaterium* in that the percent inhibition dropped to approximately 27% and 18%, respectively.

Further studies were undertaken to examine the effect post induction incubation times have on the production of Mj-AMP2 from *E. coli* AD494[pAE1]. The percent inhibition of growth of *B. megaterium* and *M. luteus* was measured using crude protein extracts that had been induced for one round of translation (1.5 minutes) prior to the addition of chloramphenicol. After the addition of chloramphenicol, the cells were incubated at 37°C for an additional 7.5, 15 or 30 minutes prior to protein extraction. From this set of experiments it was found that the presence of the crude protein extract had no effect on the growth of either *B. megaterium* or *M. luteus* (data not shown). Therefore, it is possible that after one round of translation there is not enough protein synthesized to inhibit the growth of these microorganisms and that the addition of chloramphenicol at this point in time prevents further production of Mj-AMP2.

The percent inhibition of growth of these microorganisms was also measured when cultures of *E. coli* AD494[pAE1] were exposed to chloramphenicol for one minute prior to being induced for 7.5, 15 or 30 minutes with IPTG. As shown in Figure 9, translation is not completely halted by exposing the cells to chloramphenicol for one minute prior to induction, as 22% and 10% growth inhibition of *B. megaterium* and *M. luteus*, respectively, was observed at 7.5 minutes post induction. However, as the

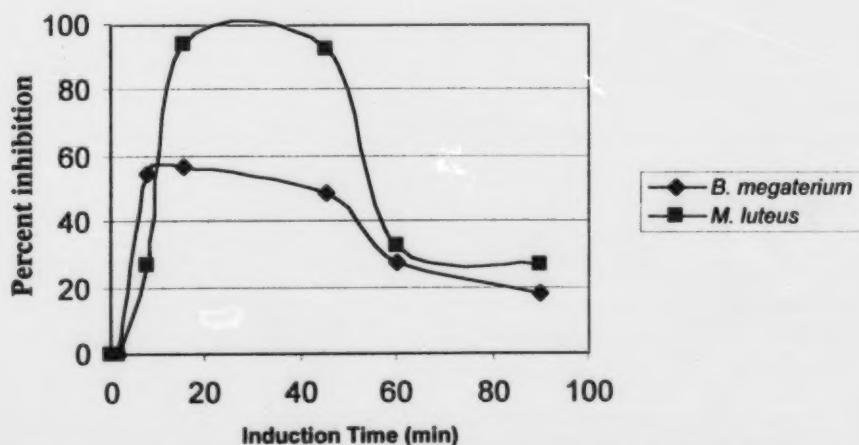


Figure 8. The effect of induction time on the inhibition of control bacterial strains. Crude protein extracts containing Mj-AMP2 were obtained from *E. coli* strain AD494[pAE1] cells after different periods of induction time with 0.2 mM IPTG. AMP extracts were then used to challenge *B. megaterium* and *M. luteus* strains.

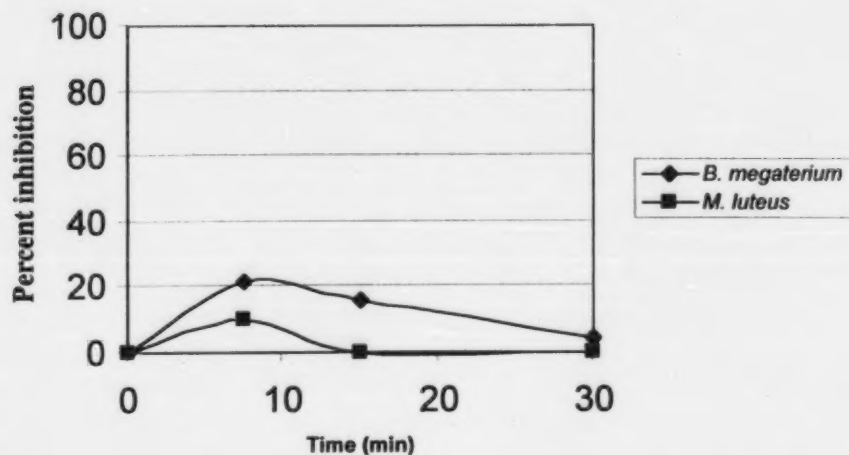


Figure 9. Percent inhibition of control bacterial strains were measured when *E. coli* cells used to obtain the crude protein extracts containing Mj-AMP2 were treated with 16mg/ml chloramphenicol for one minute prior to induction with 0.2 mM for 7.5, 15 or 30 minutes.

post induction incubation period was increased the percent inhibition of growth of both microorganisms decreased. As well, the levels of inhibition never peaked at the high levels observed when chloramphenicol was absent from the cultures suggesting that it takes time for the antibiotic to completely shut down translation.

To determine the concentration of the inducer IPTG required for the best expression of the synthetic *amp2* 100-ml cultures of AD494[pAE1] were grown to an OD₅₉₅ of 0.3-0.4 at which point they were induced with IPTG for one hour prior to protein extraction. The results observed from this study indicate that as the concentration of IPTG is increased a decrease in the AMP2-mediated inhibition occurs. From these experiments it was found that the highest percent inhibition of growth of *B.*

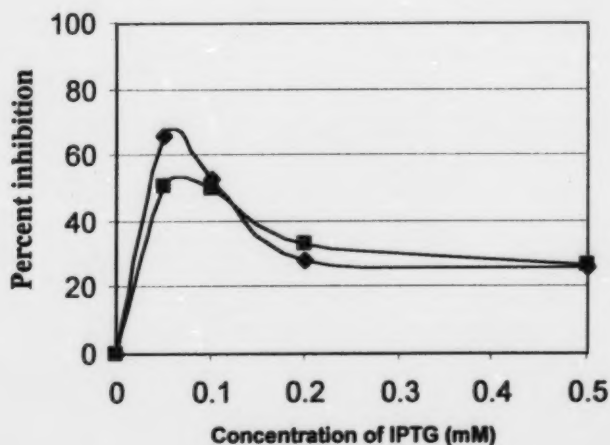


Figure 10. The effect of IPTG concentration on the inhibition of control bacterial strains. Crude protein extracts containing Mj-AMP2 were obtained from *E. coli* strain AD494[pAE1] cells that had been induced for 1 hour using different concentrations of IPTG. AMP extracts were then used to challenge *B. megaterium* and *M. luteus* strains.

megaterium and *M. luteus* occurred when 0.1 mM IPTG was used for induction (Figure 10).

Effect that cations have on growth inhibition of *B. megaterium* and *M. luteus*

Another factor thought to play a role in the ability of Mj-AMP2 peptide to inhibit microbial growth was the type of media used in each of the titre plate assays. In all of the studies previously described in this report Luria Broth (LB) was used. However, the bacterial assays undertaken by Cammue *et al.* (1992) using the Mj-AMP2 peptide purified from *M. jalapa* involved the use of 1% tryptone. As well, Cammue *et al.* (1992) reported that the activity of this peptide could be adversely affected by the presence of cations. Thus it was of interest to know whether the presence of 1% NaCl in LB significantly affected antimicrobial activity. As illustrated in Figure 11, the presence of NaCl in the media had no effect on the growth inhibition of *M. luteus* as 44% inhibition was observed in assays done in LB or 1% tryptone. When assays against *B. megaterium* were performed in LB, 25% inhibition was observed and in 1% tryptone, 14% inhibition was observed.

With the optimal conditions for the expression of Mj-AMP2 determined we have undertaken plate assays to determine if a clear zone of inhibition could be observed against the control microorganism *B. megaterium*. As shown in Figure 12, crude protein extracts containing Mj-AMP2 did cause a significant zone of clearing of approximately 15 mm.

With the *Mj-amp2* gene now producing a peptide that displays antimicrobial activity against the control microorganisms *B. megaterium* and *M. luteus* we have been able to concentrate on our objective of testing a panel of pathogens to determine which organisms are susceptible to this peptide. Preliminary testing and found that crude protein extracts containing Mj-AMP2 do have an effect on the growth of some microorganisms such as *Agrobacterium tumefaciens*, *Bacillus cereus*, *Bacillus subtilis* and *Listeria monocytogenes*. However, it does not appear to have an effect the growth of *E. coli*, or *Enterobacter aerogenes* (Table 2). While resources for continuing this project are no longer available, further testing is currently being undertaken on a number of other bacteria in order to complete this element of research.

As reported previously, we did experience difficulty obtaining clones containing antimicrobial genes that contained high levels of active antimicrobial peptides. Initially, when activity could not be observed we considered obtaining peptides from their original source (other research groups). Unfortunately, the patent laws and necessary contractual agreements were highly restrictive, hence we could not gain free access to these control compounds. Furthermore, having the peptides synthesized was not possible due to the prohibitive costs. It also became clear that most of the work performed was on either chemically-synthesized peptide compounds, or peptides extracted from actual plant material and that very little information progress had been made where genetic engineering methods had been used to produce active plant AMP. We therefore chose to perform fundamental ground-breaking research and concentrate on solving the basic research requirements for AMP expression in prokaryotic systems. Our intent was that these preliminary developments would facilitate advances in the areas applied research.

Conclusions

The work completed to date represents an essential first step for the expression of synthetic plant antimicrobial genes in prokaryotic expression systems. However, additional research remains to be conducted as we have not yet fully explored the potential for plant AMP application as antimicrobial agents against food spoilage and pathogen bacteria. Areas that require further clarification include the manipulation of engineered strains for constitutive expression of these peptide genes, as well as refinement of expression vector systems which facilitate the stability and correct conformation of the

Table 2. Percent inhibition of growth of selected foodborne pathogens incubated in the presence of 100 mg crude protein extract isolated from *E. coli* AD494 [Mj-AMP2]. The *E. coli* cells were induced with 0.1 mM IPTG for 45 minutes prior to protein extraction. The assays were incubated for 18 hours in 96-well microtiter plates at 28°C. Each assay was performed in triplicate.

Test Microorganism	Percent Inhibition
<i>E. coli</i> DH5 α	0
<i>Bacillus cereus</i>	49
<i>Bacillus subtilis</i>	29
<i>Listeria monocytogenes</i>	17
<i>Enterobacter aerogenes</i>	0

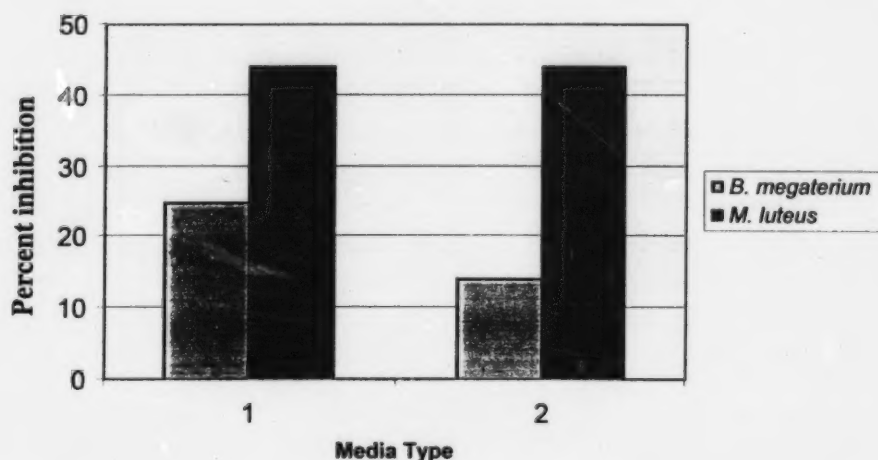


Figure 11. The effect of culture media on the inhibition of growth of *B. megaterium* and *M. luteus*. 1=LB broth and 2=1% tryptone. The cells were induced for one hour with 0.1 mM IPTG prior to protein extraction.



Figure 12. Diffusion plate assay showing the effect of Mj-AMP2 protein on the growth of a lawn of *Bacillus megaterium*. Crude protein extracts were obtained from 3 different cell treatments, including the untransformed parent *E. coli* strain (AD494), *E. coli* AD494 transformed with the expression plasmid vector pSE380 (without the peptide gene inserted), and *E. coli* transformed with AD494 that contained the *Mj-amp2* gene. An approximate 12 mm zone of inhibition was seen when the Mj-AMP2 peptide was present in the well.

peptide molecule. While there is little doubt that AMPs represent an important class of antimicrobial agent, the potential for use of plant AMPs against foodborne pathogen and spoilage bacteria remains to be explored further.

Personnel

The following list includes all the people who were involved in this project:

Dr. George Khachatourians - Dept Head and Co-PI

Dr. Darren Korber Assistant Professor and Co-PI

Karthikeyan Subramanian - Technician

Dr. Gideon Wolfaardt -Research Associate and Co-PI

Dr. Adrienne Woytowich -Postdoctoral Fellow

There were some personnel-related issues which impacted the timing of this research to a degree. Briefly, this included the loss of a key group member to a faculty position in Africa, as well as the maternity leave of our PostDoctoral Fellow.

Equipment

No equipment was purchased or rented using funds provided by this grant.

Project Developed Materials

Due to the difficulties encountered in producing a peptide with antimicrobial activity we have only now recently enough information for what we feel would be a worthy publication. We will be presenting a poster at the upcoming Annual Canadian Society of Microbiology Conference in June. ADF will be acknowledged for their support in this publication.

Project Photos

Nil

Acknowledgment

ADF will be acknowledged in all presentations, posters and publications resulting from this research.

Expense Statement

Attached, please find the project's Final Statement of Expenditures, and accompanying invoice for the final period, as prepared by the Division of Financial Services, University of Saskatchewan.

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Appendix A

One letter codes for amino acids

A	alanine
R	arginine
N	asparagine
D	aspartic acid
C	cysteine
E	glutamic acid
Q	glutamine
G	glycine
H	histidine
I	isoleucine
L	leucine
M	methionine
F	phenylalanine
P	proline
S	serine
T	threonine
W	tryptophan
Y	tyrosine
V	valine